

Detection of Rabies Virus Antigen or Antibody Using Flow Cytometry

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Background: Rabies is invariably a fatal encephalomyelitis that is considered to be a serious public health problem. Rabies diagnosis must be rapid and conclusive. Detection and quantification of antirabies antibodies is used for assessment of the effectiveness of rabies vaccines. Hence, computer-automated detection of fluorescence using flow cytometry was attempted to reduce the work time required to undertake the conventional rapid fluorescent focus inhibition test (RFFIT).

Methods: Pasteur virus (PV)-infected mouse neuroblastoma (MNA) cells were stained with rabies virus antinucleocapsid antibody, fluorescein isothiocyanate (FITC) conjugate, and the percentage of infected cells at 24, 48, and 72 h postinfection (PI) was determined using flow cytometry. Serum samples containing known antibody titres estimated by RFFIT in terms of IU/ml were used to neutralize 50 FFD₅₀ dose of PV. The percentage of MNA cells infected by the un-neutralized virus was estimated by flow cytometry. Using the value of the percentage of cells infected in the presence of known negative serum as 100%, the infection inhibition caused by antibodies at each dilution of positive reference serum was calculated and a regression equation generated for the prediction of rabies virus antibody titres in test sera samples as equivalent units per ml (EU/ml).

Results: There was a significant increase in the percentage of infected cells between 24 and 48 h PI from 26.45 to 75.28%. The percentage of cells having high side scatter was also highest at 72 h PI (11.11%). Antibody titres predicted by flow cytometry and those estimated by RFFIT as IU/ml showed a correlation coefficient of 0.74.

Conclusions: Thus, flow cytometry could be used to detect rabies virus antigen in infected cells and to predict serum antibody titres from a single dilution of serum tested with the potential advantages of automation, rapidity, and lack of subjectivity. It has the potential to replace the time-tested RFFIT in rabies serology in the years to come. © 2006 International Society for Analytical Cytology

Key terms: rabies virus; PV strain; flow cytometry; RFFIT; equivalent units; side scatter

Rabies is an acute neurological disease caused by rabies virus belonging to the family Rhabdoviridae and genus Lyssavirus. It is manifested as fatal encephalitis and protection has been achieved by pre- or more frequently postexposure vaccination (1). In the past, histopathological methods for detection of virus inclusions, the Negri bodies (2), mouse inoculation test (3), and direct fluorescent antibody test (FAT) (4) were used for the detection of rabies virus or antigen. But in some cases, virus isolation was necessary to enhance the sensitivity of virus detection (5).

The mouse neuroblastoma (MNA) cells are widely used for rabies virus propagation and assay, since they share a number of characteristics with human neurons (6). Moreover, they proved to be more sensitive than

BHK₂₁ cells for isolation of street rabies virus (7). Virus infection in cell culture was normally detected by immunofluorescence, since there is no observable cytopathic effect elicited by the virus. However, accurate quantification of rabies virus infected cells was not possible by this method, which may be helpful to study the infection process further.

Detection and quantification of antirabies virus antibodies is useful for checking immunity to rabies virus or

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assessment of effectiveness of rabies vaccines (8). Although diagnosis of rabies is generally accomplished by detection of the virus/antigen in tissues or body fluids, there is a need for rapid, reliable, and reproducible serological test that could be used in animal population survey, evaluation of vaccines, prophylaxis as well as supplementing antigen detection methods (9). Detection and quantification of rabies virus antibodies is being done by various methods such as mouse neutralization test (10), in vitro neutralization assay (11), enzyme-linked immunosorbent assay (12,13), fluorescent antibody virus neutralization test (14), and rapid fluorescent focus inhibition test (RFFIT) (15). Of all the above methods, RFFIT has been in practice for the past 30 years and recommended by the World Health Organization (WHO) expert committee on rabies (16). However, there is an element of subjectivity in RFFIT during assessment or counting of the fluorescent foci of virus infection, and it is highly time consuming and cumbersome.

Nowadays, new techniques are being introduced to make virological and serological diagnosis more accurate. One such technique is flow cytometry. It has emerged as a method of choice for automated analysis of cells in suspension and permitted rapid analysis at the level of single particles by optical means based upon LASER activation of fluorescent dyes (17). Flow cytometry has been widely used for detection of several viruses such as rotavirus (18), herpes simplex virus (HSV) (19), dengue virus (20), Cytomegalo virus (CMV) (21), measles virus (22), and vesicular stomatitis virus (23). This technique has also been employed for the detection and quantification of antibodies against viruses such as human immunodeficiency virus (24), CMV, and HSV (25). There is one previous report of the successful application of flow cytometry in the detection of intracellular rabies virus antigen (5) or antibodies (26). It was suggested by these authors that more such studies comparing flow cytometry with other established methods and the use of MNA cells (in place of BHK₂₁ cells) would be beneficial before more extensive application of this new approach in clinical laboratories (5). Woldehiwet (16) also called for the application of computer-automated detection of fluorescence methods for rapid and efficient titration of anti-rabies antibodies in immunized hosts to reduce the work time required to undertake RFFIT. Hence, this study was undertaken to evaluate the application of flow cytometry for the detection of rabies virus antigen in MNA cells and predictive quantification of rabies virus antibodies in field sera samples.

MATERIALS AND METHODS

Cells and Virus

MNA cells were cultured in Eagle's minimum essential medium (MEM) (Sigma Chemical Company, USA) supplemented with MEM vitamin solution (Gibco BRL, USA) and antibiotics (final concentrations of benzyl penicillin 100 IU and streptomycin 100 µg/ml). Confluent monolayers were trypsinized with 0.1% trypsin-EDTA solution.

The Pasteur virus (PV), available at Rabies Unit, Department of Animal Biotechnology, Madras Veterinary College, Chennai, India, was diluted at the level of 50% focus forming dose (50 FFD₅₀) as per standard procedure (27). 50 FFD₅₀ of PV strain of rabies virus was used in all experiments.

Serum Samples

Thirty-five serum samples were collected from rabies virus vaccinated or unvaccinated dogs that reported at Madras Veterinary College Hospital, Chennai, India, heat inactivated at 56°C for 30 min, and stored at -20°C until use. Positive reference serum (Indian Immunologicals Private Ltd., Hyderabad, India) having RFFIT titre of 64.56 IU/ml was diluted to contain 2 IU/ml and used in parallel in RFFIT. Four known positive reference sera samples having antibody titres of 10.38, 10.38, 12.81, and 12.81 IU/ml (Indian Immunologicals Private Ltd.) were used in flow cytometry assay to formulate the regression equation. A known negative serum having no RFFIT titre (Indian Immunologicals Private Ltd.) was used as a control in flow cytometry analysis for antibody quantification.

Fluorescent Antibody Test

MNA cells (5×10^5 cells per ml) were infected with PV (50 FFD₅₀) and subjected to FAT as described elsewhere (28) after 24, 48, and 72 h PI. The results of FAT were expressed as the number of fields showing the presence of infected fluorescent foci out of 20 fields observed at 200× magnification in a fluorescent microscope (Nikkon, Japan).

RFFIT

Rabies virus antibodies were measured in RFFIT following the method described by Smith et al. (15). Briefly, serum was diluted 5-fold from 1:5 and each dilution was neutralized with 50 FFD₅₀ of PV at 37°C for 45 min. Then, 5×10^5 MNA cells were added and incubated at 37°C for 24 h at 5% CO₂ in Labtek 8-well slides. The cultures were fixed with 75% acetone for 10 min at 4°C and allowed to react with fluorescein isothiocyanate (FITC) conjugated rabies antinucleocapsid antibody (BioRad, USA). Following washing, the number of fields with infected fluorescent foci out of 10 fields counted was calculated for each serum dilution. Then, the end-point titres were estimated using the method of Reed and Muench (29). The international units (IU) of antibody per milliliter was further determined using a standard serum whose IU/ml was already known.

Flow Cytometry Assay

Sample processing for intracellular rabies virus antigen detection. MNA cells in 25 cm² tissue culture flasks were infected with 500 µl of 50 FFD₅₀ of PV and maintained in the humidified atmosphere containing 5%

CO₂ at 37°C. The kinetics of rabies virus infection was determined by harvesting both infected and uninfected cells at 24, 48, and 72 h PI. The trypsinized cell count was adjusted to 2.5×10^6 cells per ml and washed twice (1,000 rpm for 5 min at 4°C) with cold phosphate buffered saline (PBS). The washed cell pellet was resuspended in 100 µl of Cytotfix/Cytoperm solution (BD Pharmingen, USA) and incubated at 4°C for 20 min for permeabilization. Then, cells were washed once with cold staining solution (PBS containing 1% bovine serum albumin, 0.01% sodium azide, and 0.1% saponin) and the pellet from the last wash was resuspended in 100 µl of rabies virus antinucleocapsid antibody conjugate (BioRad, USA) to which 0.1% saponin was added and incubated for 20 min in the dark at 4°C. Cells were washed once with 1 ml of cold staining solution at 1,000 rpm for 5 min at 4°C. The final cell pellet was resuspended in 500 µl of FACS flow buffer and stored at 4°C until used for flow cytometry.

Flow cytometry analysis for rabies virus antigen detection. A flow cytometer (FACSCalibur, Becton Dickinson, USA) equipped with a 15 mV and 488 nm argon ion LASER was used to analyze the cell infection kinetics. The forward scatter detector was set at E01 with an amp gain of 1.49 and a threshold value of 200. The side scatter (SSC) was set at 501 V with an amp gain of 3.33. A total of 10,000 events were recorded for each sample. Percentage of infected and uninfected cells were analyzed in quadrant dot plot using CELL QUEST software with fluorescent lamp1 (FL1) as an *x*-axis parameter and SSC as a *y*-axis parameter. The FL1 was set at 469 V. These parameters placed 97–98% of the uninfected cells treated with conjugate within the lower left (LL) or upper left (UL) quadrants in the dot plots. The infected cells treated in the same manner were analyzed without alterations in any of the parameters set.

Virus neutralization in cell culture. Five-hundred microliter of an optimal predetermined sample dilution (1:25) were added in sterile 1.5 ml microfuge tubes and mixed with equal volumes of 50 FFD₅₀ of PV virus. The mixture was then incubated for 45 min at 37°C and 5% CO₂ tension for neutralization. Then, the virus-sera mixture was plated in 24-well tissue culture plates with 1×10^6 MNA cells per well and incubated for 2 h. Medium was changed after 2 h to avoid serum toxicity to the cells and the cells were incubated for an additional 22 h.

Flow cytometry analysis for determination of rabies virus antibodies titres. Cell monolayers were disrupted by trypsinization and the detached cells were recovered and washed twice with cold PBS (4°C) and stained with the antinucleocapsid conjugate as detailed earlier. The percentage of cells infected with unneutralized virus was analyzed in the dot plot with FL1 as *x*-axis parameter and SSC as *y*-axis parameter. The percentage of cell population in the lower right (LR) and upper right (UR) quadrants were calculated, which defined the percentage of infected cells with unneutralized virus antigen. The reduction in infected cell population

after neutralization was analyzed in terms of percentage of infection inhibition for which the percentage of infected cells with negative serum was taken as 100% for each trial. Appropriate controls were run during each trial.

Experimental Design

Experiment I: detection of intracellular rabies virus antigen in cell culture by FAT and flow cytometry assay. MNA cells were infected with 50 FFD₅₀ of PV strain of rabies virus. The cell infection kinetics was analyzed after 24, 48, and 72 h PI by both FAT and flow cytometry. Results for FAT were expressed as the number of fields showing the presence of infected foci out of 20 fields observed. For flow cytometry, the results were expressed as percentage of infected cells at each time period. The percentages of cells showing high SSC were also calculated.

Experiment II: generation of regression equation using known positive reference sera. Four positive reference sera were diluted to contain 1.28–0.02 IU/ml and each dilution was used to neutralize 50 FFD₅₀ of PV strain of rabies virus. MNA cells infected with unneutralized virus were analyzed by flow cytometry after 24 h. The percentage of infection inhibition due to the neutralization of virus by each dilution of the reference serum was obtained by comparing with percentage of infected cells with negative serum (100%). Correlation between percentage of infection inhibition obtained from the serial dilution of four positive reference sera samples and their corresponding RFFIT titres in IU/ml were analyzed. Out of the four samples, the sera sample having highest correlation coefficient was used to draw the regression line. The antibody titres of the test sera samples were predicted as EU/ml using the generated regression equation.

Experiment III: assessment of rabies virus antibody titres in EU/ml using flow cytometry. Totally, 35 dog sera samples were diluted to the predetermined single dilution (1:25) and used to neutralize 50 FFD₅₀ of PV strain of rabies virus. Percentage of cells infected by unneutralized virus was analyzed using flow cytometry after 24 h. The percentage of infection inhibition was calculated and substituted in the regression equation and their titres predicted as EU/ml.

Data Analysis

Regression and correlation between percentage of infection inhibition obtained from the serial dilution of four positive reference sera samples and their corresponding RFFIT titres in IU/ml were analyzed using MS Excel package. The slope and intercept of the regression line was calculated and the regression equation was formulated as $Y = mX + c$. The *Y* value, which defined the antibody titres of test samples, was predicted by substituting the percentage of infection inhibition of the test

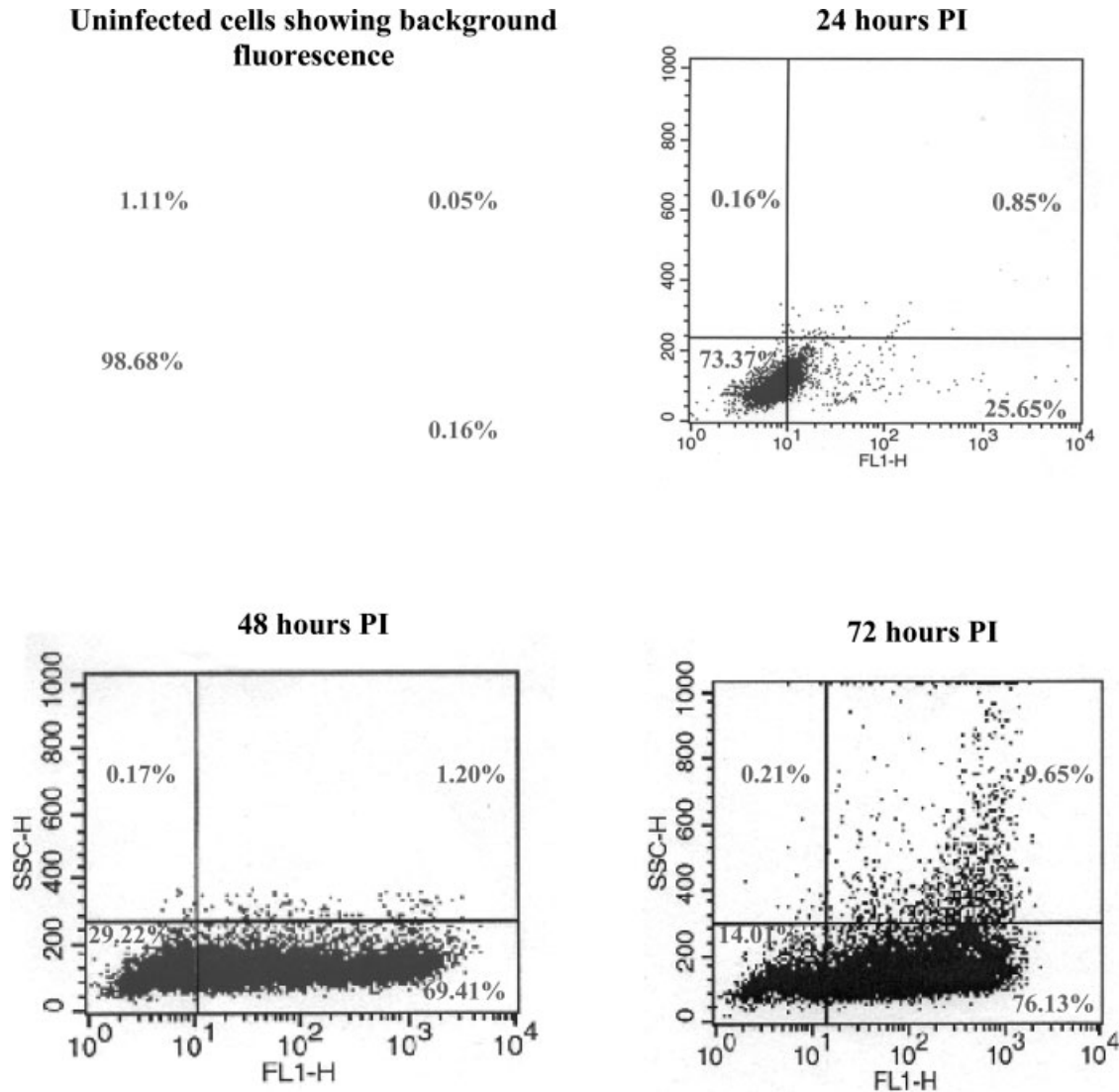


FIG. 1. MNA cells infected with 50 FFD₅₀ of PV strain of rabies virus and the percentages of virus-infected cells analyzed by flow cytometry at different hours of PI. Quadrant description: upper left (UL), granular cells with no fluorescence; upper right (UR), granular cells with FITC fluorescence; lower left (LL), cells with no granularity or fluorescence; lower right (LR), cells with no granularity but with FITC fluorescence; x-axis parameter (FL1-H) indicates intensity of FITC fluorescence in log scale; y-axis parameter (SSC-H) indicates granularity of cells in linear scale.

serum samples in the regression formula, and after multiplication by the serum dilution factor of 25, it was expressed as EU/ml. Correlation between EU/ml of anti-

body titres predicted and their corresponding RFFIT titres in IU/ml of the same serum samples was analyzed using MS Excel package.

Table 1
Percentage of Infection and Percentage of Infection Inhibition Obtained by Rabies Virus Neutralization Using Different Dilutions of a Representative Positive Reference Sera

Sample	Dot plot analysis		% infection	% infection inhibition
	% infected cells	% uninfected cells		
Infected cells ^a + negative sera (0 IU/ml)	28.57	71.43	100	0
Infected cells + 0.02 IU/ml	29.99	70.01	100	0
Infected cells + 0.08 IU/ml	24.97	75.03	87.39	12.61
Infected cells + 0.32 IU/ml	21.53	78.47	75.35	24.65
Infected cells + 1.28 IU/ml	16.67	83.33	58.34	41.66

^aMNA cells infected with 50 FFD₅₀ of PV strain of rabies virus.

Table 2
Correlation Between the Antibody Titres of Four Positive Reference Sera in IU/ml and Their Corresponding Percentage Infection Inhibition Assessed by Flow Cytometry

IU/ml of the diluted positive reference serum	Percentage of infection inhibition			
	Positive sera 1 (10.38 IU/ml)	Positive sera 2 (10.38 IU/ml)	Positive sera 3 (12.38 IU/ml)	Positive sera 4 (12.38 IU/ml)
0.02	0	0	0	0
0.08	21.1	15.6	15.38	12.61
0.32	38.3	45.4	23.96	24.65
1.28	58.3	56.7	38.76	41.66
Correlation coefficient	0.89	0.83	0.89	0.92

RESULTS

Experiment I

In FAT, only five to six fields were found to contain positive fluorescent foci out of 20 fields counted after 24 h PI. All the 20 fields examined contained positive fluorescent foci after 48 and 72 h PI. No fluorescent foci were seen in control-uninfected cells. The mean \pm SE percentages of virus-infected cells analyzed by flow cytometry at 24 h PI was low (26.45 ± 0.14) and at 48 and 72 h PI, it was 75.28 ± 4.67 and 84.43 ± 0.93 respectively ($n = 4$). A representative dot plot is shown in Figure 1. The geometric mean fluorescence values assessed, using single parameter histogram statistics, revealed a mean (\pm SD) values of 73.31 ± 17.7 , 80.02 ± 9.2 , 145.47 ± 11.6 , and 159.02 ± 3.6 for control, 24, 48, and 72 h PI cultures, respectively. The mean \pm SE percentages of infected cells with high SSC values (UR quadrant) gradually increased during the course of virus replication in MNA (Fig. 1). A highest mean percentage value of 11.11 ± 0.81 was observed at 72 h PI.

Experiment II

Four positive dog sera samples having RFFIT antibody titres of 10.38, 10.38, 12.38, and 12.38 IU/ml respectively were diluted to contain 1.28–0.02 IU/ml and used to neutralize 50 FFD₅₀ of virus. Percentage of infection inhibition of serial dilutions of a representative positive serum is shown in Table 1. The correlation coefficient between the percentage of infection inhibition and the RFFIT titres in IU/ml of the four positive reference sera samples are summarized in Table 2. Highest correlation coefficient of 0.92 was obtained with positive serum no. 4 and hence this was used for regression analysis. Value of slope (m) and intercept (c) of the regression line was found to be 0.0304 and -0.173 respectively. On the basis of these values, the regression equation was set as $Y = 0.0304 X - 0.173$ (Fig. 2).

Experiment III

Percentage of infection inhibition for test sera was calculated by comparing with the percentage of virus-in-

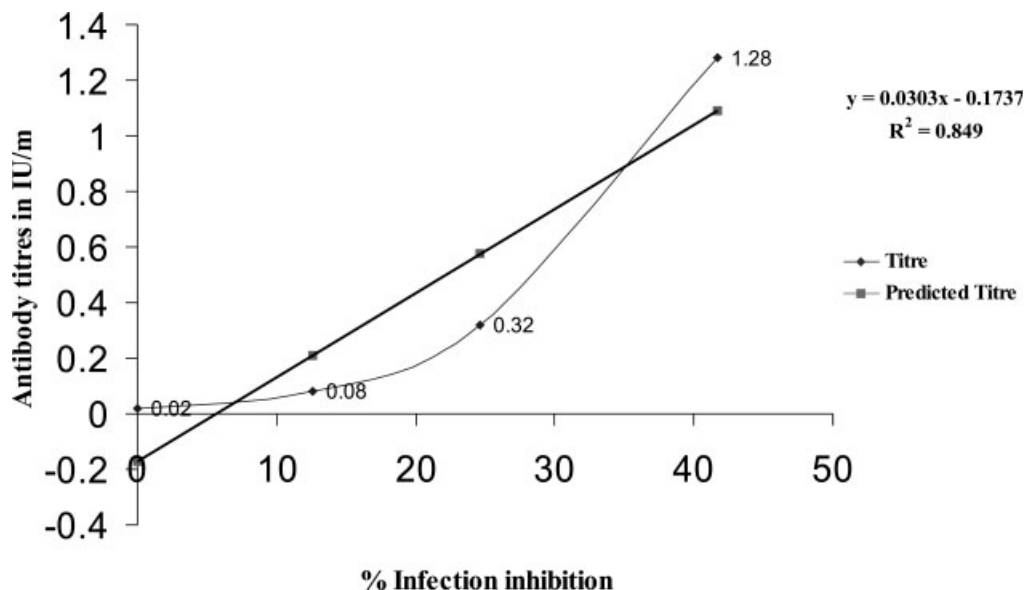


FIG. 2. Formation of regression line and regression equation using known representative positive reference serum no.4 to predict antibody titres in EU/ml.

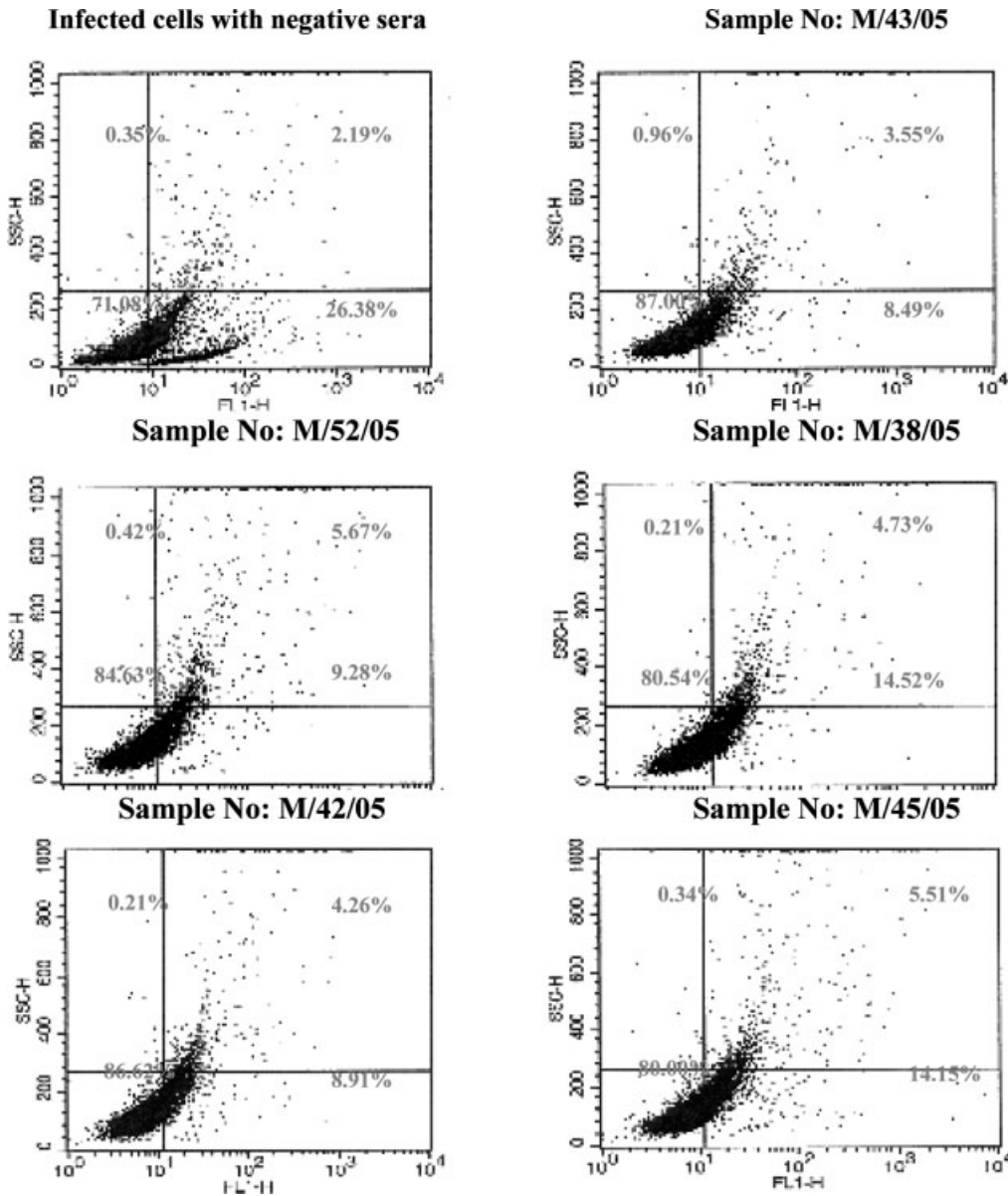


FIG. 3. Dot plot analysis of virus neutralization using different dog sera samples showing reduction in percentage of infected cells analyzed by flow cytometry. Sum of percentage of cells in the upper right (UR) and lower right (LR) quadrants of this dot plot indicates the percentage of infected cells showing FITC fluorescence (infected cells). Note the reduction in the percentage of these cells after neutralization with test sera (in comparison with infected cells incubated with negative serum). Other legends as shown in Figure 1.

infected cells with known negative serum taken as 100% and were substituted in regression equation and the titres were predicted and expressed as EU/ml. A representative flow cytometry dot plot results of five test sera samples are shown in Figure 3 and calculation of their predicted flow cytometry titres is given in Table 3. Correlation coefficient of antibody titres expressed in EU/ml for the test sera samples and their RFFIT titre in IU/ml was found to be 0.74 and is shown in Figure 4.

DISCUSSION

Diagnosis of rabies virus antigen is conventionally done by Negri body examination or by direct FAT using rabies

antinucleocapsid antibody conjugate on brain impression smears. FAT is considered to be a standard rabies virus diagnostic test against which other diagnostic approaches need to be compared (30). Cultivation of virus in cell culture allows the replication of the rabies virus originally present in the sample and thus would enhance the sensitivity of virus detection over direct FAT done on impression smears. Further, for the performance of RFFIT, a predetermined dose of virus (50 FFD₅₀) grown in MNA or BHK₂₁ cells is required. For the above reasons, it is imperative that rabies virus needs to be cultivated in cell culture and antigen demonstrated by suitable methods.

Table 3
Calculation of Predicted Flow Cytometry Titres in EU/ml From Percentage of Infection Inhibition Obtained by Neutralization of 50 FFD₅₀ of PV With Different Dog Sera Samples

Sample no.	Dot plot analysis		% infection	% infection inhibition	Predicted titres (EU/ml)	Predicted titres (EU/ml) × dilution factor (25)
	% infected cells (UR + LR)	% uninfected cells (UL + LL)				
Infected cells with negative sera	28.57	71.43	100	0	–	–
M/43/05	12.04	87.96	42.14	57.86	1.579458	39.48645
M/52/05	14.95	85.05	52.32	47.68	1.271004	31.7751
M/38/05	19.25	80.75	67.37	32.63	0.814989	20.37473
M/42/05	13.17	86.83	46.09	53.91	1.459773	36.49433
M/45/05	19.66	80.34	68.81	31.1	0.76863	19.21575

The RFFIT for rabies virus antibody assay involves neutralization of the test serum with fixed amount of rabies virus followed by detection of the unneutralized virus by FAT following inoculation in susceptible cell cultures such as MNA or BHK₂₁ cells. Thus, FAT has been the most commonly used and approved method for the detection of both, rabies virus antigen from suspected samples and unneutralized virus in antibody assays.

Flow cytometry is a technology that simultaneously measures and then analyzes multiple physical characteristics of single cells, such as relative size, relative granularity, or internal complexity, and fluorescence intensity using an optical to electronic compiling system that records how the cell or particle scatters incident LASER light and emits fluorescence as they flow in a fluid stream through a beam of light. Sensitivity to detect even less intensity of fluorescence, higher objectivity through automation, and the ability to count more than 10,000 cells within 5–10 min are the potential advantages of flow cytometry.

In the earlier context, flow cytometry could be a very rapid, objective, and useful tool for application in rabies

virology that has always depended upon the conventional fluorescence-based methods. The drawbacks of FAT include subjectivity and manual observation of stained fluorescent foci of infection, which is highly cumbersome and time consuming. Thus, the application of flow cytometry would overcome the difficulties encountered in the conventional FAT.

In the present study, flow cytometry was initially applied to antigen detection and compared with the conventional FAT. By this method, it was found that at 48 and 72 h PI, 100% of fields have infected fluorescent foci. However, accurate quantification was possible only using flow cytometry, wherein $84.43 \pm 0.93\%$ of cells could be shown to be infected after 72 h PI. There was a significant increase in the percentage of infected cells from 24 h (26.45 ± 0.14) to 48 h PI (75.28 ± 4.67). In addition, the mean fluorescent values also showed a dramatic increase from 24 to 48 h PI along with the percentages of infected cells. This pattern was also seen in the FAT although such accurate estimation was not possible. A similar study carried out by Bordignon et al. (26) in which PV and wild rabies virus were used to infect C₆ and BHK₂₁ cells revealed that 4.1–7.1% of the C₆ and

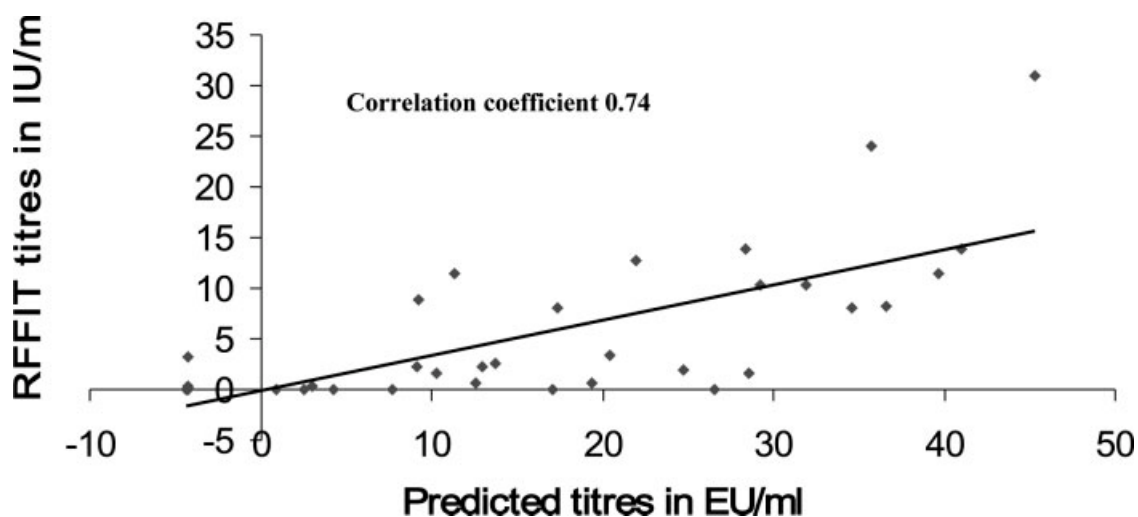


Fig. 4. Correlation between RFFIT titres in IU/ml and predicted flow cytometry titre in EU/ml for dog test sera samples ($n = 35$). Each dot may represent more than one sample.

BHK₂₁ cells were infected after 12 h PI, while 71 and 81% of BHK₂₁ cells were infected with PV and wild rabies virus after 72 h PI respectively.

Another interesting observation was that the mean (\pm SE) percentage of cells having a higher SSC increased from 0.39 ± 0.14 at 24 h PI to 11.11 ± 0.81 at 72 h PI. SSC is the measure of the internal complexity or granularity of the cells. Cells having higher SSC are more granular. It can be postulated that in rabies virus infected cells, the fluorescence is in the form of "sand dust" like particles, which are thought to be smaller sized Negri bodies. The presence of such particles would have increased the granularity of the cells and leading to increase in SSC. The observation might be of value for the identification of rabies virus infected cells even in the absence of fluorescence labeled antinucleocapsid antibody conjugate, though not confirmatory.

Intracellular antigen detection using flow cytometry involves permeabilization of the live cell membrane using appropriate detergents to enable fluorescence-tagged antibody to bind to the specific antigen present in the cytoplasm of the infected cells. The cells should still be live so that enumeration using flow cytometry can be done. Several authors have used different permeabilization reagents such as acetone-methanol-formalin (1:1:1) (18), paraformaldehyde and methanol (31), FACS lysing solution (5), 0.1% saponin (20), and Cytoperm/Cytofix solution (32). Initial attempts using FACS lysing solution, which was already used to study rabies virus antigen and antibody in flow cytometry (5,25), proved unsuccessful in our hands. The use of proprietary reagent called Cytoperm/Cytofix (BD Pharmingen, USA) coupled with addition of saponin to the rabies virus antinucleocapsid conjugate gave us good results. Permeabilization, being a reversible process, has to be maintained during all the steps of fluorescence labeling of nucleocapsid antigen. In this study, we got optimum results only when this factor was ensured. While handling a zoonotic, fatal virus such as rabies virus, it has to be ensured that infective aerosols are not generated during flow cytometry examination. The Cytoperm/Cytofix solution contains formaldehyde that would kill rabies virus. Infect formalin has been used earlier for inactivating rabies virus for preparation of inactivated rabies virus vaccines.

The correlation coefficient for the four different positive reference sera was in the range of 0.83–0.92, indicating good correlation between the estimated percentage infection inhibition and the IU/ml of the positive reference sera. The correlation between RFFIT titre in IU/ml and flow cytometry titres in EU/ml was 0.74. Using the standard curve approach, Bordignon et al. (26) reported a correlation coefficient of 0.77 between RFFIT and flow cytometry titres. The regression method was adopted to get EU/ml of test sera samples, since it involves simple mathematical calculations and EU/ml could also be predicted rapidly once the percentage of infection inhibition of that sera sample was available. Only limited number of sera samples was analyzed using flow

cytometry for the determination of antibody titres in terms of EU/ml. The correlation between EU/ml and IU/ml in RFFIT may increase if several hundreds of samples are tested.

Thus, the present study exemplifies the great potential flow cytometry offers in rabies virus serology. For laboratories handling hundreds of samples, this method would prove ideal, since it does not involve visual screening of fluorescent foci of infection under the microscope.

CONCLUSION

The automated flow cytometry assay was found to be useful in assessing the exact percentage of rabies virus infected cells unlike conventional FAT and allowed predictive quantification of rabies virus antibodies from a single serum dilution without observer bias. The correlation coefficient between the predicted flow cytometry titres in EU/ml and the observed RFFIT titres in IU/ml was found to be 0.74. Thus, flow cytometry can be applied in laboratories handling large number of samples thereby avoiding the cumbersome procedures of visual microscopic screening needed for RFFIT.

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LITERATURE CITED

1. Sehgal S. Medical and veterinary aspects of rabies prevention and control. In: Dobet B, Meslin FX, editors. Proceedings of third international symposium on rabies control in Asia. Paris: Meslin Elsevier; 1997. p 140–144.
2. Tierkel ES, Ataunasiu P. Rapid microscopic examination of negri-bodies and preparation of specimens for biological tests. In: Meslin FX, Kapalan MM, Koprowski H, editors. Laboratory Techniques in Rabies, 4th edition. Geneva: World Health Organization; 1996. p 55–62.
3. Koprowski H. The mouse inoculation test. In: Meslin FX, Kapalan MM, Koprowski H, editors. Laboratory Techniques in Rabies, 4th edition. Geneva: World Health Organization; 1996. p 80–87.
4. Goldwasser RA, Kissling RE. Fluorescent antibody staining of street and fixed rabies vaccine antigens. Proc Soc Exp Biol Med 1958;98: 219–223.
5. Bordignon J, Ferreira SCP, Caporale GMM, Canieri ML, Kotait I, Lima HC, Zanetti CR. Flow cytometry assay for intracellular rabies virus detection. J Virol Methods 2002;105:181–186.
6. Clark HF. Rabies virus increase in virulence when propagated in neuroblastoma cell culture. Science 1978;199:1072–1075.
7. Rudd RJ, Trimarchi CV. Comparison of sensitivity of BHK₂₁ and Murine neuroblastoma cells in the isolation of a street strain rabies virus. J Clin Microbiol 1987;25:1456–1458.
8. Ondrejko A, Ondrajeka SR, Benisek Z, Frank R, Svrcek S, Madar M, Bugarsky A. Comparison of the detection and quantification of rabies antibodies in canine sera. Vet Med 2002;47:218–221.
9. Heberling RL, Kalter SS, Smith JS, Hilderbrand DG. Serodiagnosis of rabies virus by Dot immunobinding assay. Am Soc Microbiol 1987;25: 1262–1264.
10. Webster LT, Dawson JR. Early diagnosis of rabies by mouse inoculation; measurement of host immunity to rabies by mouse protection test. Proc Soc Exp Biol Med 1935;32:570–573.
11. Trimarchi CV, Rudd RD, Safford M, Jr. An in vitro virus neutralization test for rabies antibody. In: Meslin FX, Kapalan MM, Koprowski H, editors. Laboratory Techniques in Rabies, 4th edition. Geneva: World Health Organization; 1996. p 193–199.

12. Grassi M, Wandeler AI, Peterhans E. Enzyme linked immunosorbent assay for determination of antibodies to the envelope glycoprotein of rabies virus. *J Clin Microbiol* 1989;27:899-902.
13. Esterhuysen JJ, Prehaud C, Thomson GR. A liquid phase blocking ELISA for the detection of antibodies to rabies virus. *J Virol Methods* 1995;51:31-42.
14. Cliquet F, Aubert M, Sagne L. Development of a fluorescent antibody virus neutralization test (FAVN test) for the quantitation of rabies-neutralizing antibodies. *J Immunol Methods* 1998;212:79-87.
15. Smith JS, Yager PA, Baer GM. A rapid reproducible test for determining rabies neutralizing antibody. *Bull World Health Organ* 1973;48:535-541.
16. Woldehewet Z. Clinical laboratory advances in the detection of rabies virus. *Clin Chem Acta* 2005;351:49-63.
17. Alvarez-Barrientos A, Arroyo J, Canton R, Nombela C, Perez MS. Applications of flow cytometry to clinical microbiology. *Clin Microbiol Rev* 2000;13:167-195.
18. Abad FX, Pinto RM, Bosh A. Flow cytometry detection of infectious rotaviruses in environmental and clinical samples. *J Clin Microbiol* 1998;64:2392-2396.
19. McSharry JJ, Costantivo R, McSharry MB, Venezia RA, Lehman JM. Rapid detection of Herpes simplex virus in clinical samples by flow cytometry after amplification in tissue culture. *J Clin Microbiol* 1990;28:1864-1866.
20. Sydow FFOV, Santiago MA, Neves-souza PC, Cerqueira DIS, Gouvea AS, Lavotori MFH, Bertho AL, Kubelka CE. Comparison of Dengue infection in human mononuclear leukocytes with mosquito C6 /36 and mammalian vero cells using flow cytometry to detect virus antigen. *Memento Instituto Oswaldo Cruz* 2000;5:483-489.
21. Schols D, Snoeck R, Neyts J, De clerk E. Detection of immediate early, early and late antigens of human cytomegalovirus by flow cytometry. *J Virol Methods* 1989;26:247-254.
22. Lydy SL, Campans RW. Role of cytoplasmic domains of viral glycoproteins in antibody-induced cell surface mobility. *J Virol* 1993;67:6289-6294.
23. Whitt MA, Rose JK. Fatty acylation is not required for membrane fusion activity or glycoprotein assembly in the VSV variant. *Virology* 1991;185:875-878.
24. Sligh JM, Roodman ST, Tsai CC. Flow cytometric indirect immunofluorescence assay for detection of antibodies to human immunodeficiency virus (HIV). *Am J Clin Pathol* 1989;91:210-214.
25. McHugh TM, Miner RC, Logan LH, Sites DP. Simultaneous detection of antibodies to cytomegalovirus and herpes simplex virus by using flow cytometry and a microsphere based fluorescence immunoassay. *J Clin Microbiol* 1988;26:1957-1961.
26. Bordignon J, Comin F, Ferreira SCP, Caporale GMM, Filho JHCL, Zanett CR. Calculating rabies virus neutralizing antibodies titers by flow cytometry. *Reverso Instituto Medico Tropico Suo Paulo* 2002;44:151-154.
27. Smith JS, Yager PA, Baer GM. A rapid fluorescent focus inhibition test (RFFIT) for determining rabies virus neutralizing antibody. In: Meslin FX, Kaplan MM, Koprowski H, editors. *Laboratory Techniques in Rabies*, 4th edition. Geneva: World Health Organization; 1996. p 181-192.
28. Dean DJ, Abelseth MK, Atanasiu P. The fluorescent antibody test. In: Meslin FX, Kaplan MM, Koprowski H, editors. *Laboratory Techniques in Rabies*, 4th edition. Geneva: World Health Organization; 1996. p 88-95.
29. Reed LJ, Muench H. A simple method for estimating fifty percent end points. *Am J Hyg* 1938;3:493-497.
30. Bourhy H, Rollin PE, Vincent J, Sureau P. Comparative field evaluation of the fluorescent-antibody test, virus isolation from tissue culture and enzyme immuno diagnosis for rapid laboratory diagnosis of rabies. *J Clin Microbiol* 1989;27:519-523.
31. Imbert-Marcille BM, Coste-Burel M, Robillard N, Foucaud-Gamen J, Billaudel S, Drouet E. Sequential use of paraformaldehyde and methanol as optimal conditions for the direct quantification of ZEBRA and Rta antigens by flow cytometry. *Clin Diagn Lab Immunol* 2000;7:206-211.
32. Lonsdale R, Pare MG, Oerlemans M, Ophorst N, Vroys A, Havenga M, Goudsmit J, Uytendaele F, Marzio G. A rapid method for immunotitration of influenza viruses using flow cytometry. *J Virol Methods* 2003;110:67-71.